

CARROT ANTIFREEZE POLYPEPTIDESTechnical Field of the Invention

- 5 The invention relates to anti-freeze polypeptides (AFPs) and food product containing AFPs.

Background to the Invention

- 10 Anti-freeze polypeptides (AFPs) have been suggested for improving the freezing tolerance of foodstuffs.

For the purpose of the invention, the term AFP has the meaning as well-known in the art, namely those proteins which  
15 inhibit the growth of ice-crystals. See for example US 5,118,792.

WO 90/13571 discloses antifreeze peptides produced chemically or by recombinant DNA techniques from plants. The AFPs can  
20 suitably be used in food-products such as ice-cream. Example 3B shows modified ice-crystal shapes if a water-ice mixture is frozen into a film in combination with 0.01 wt% of AFP.

WO 92/22581 discloses AFPs from plants which can be used for  
25 controlling ice crystal shape in ice-cream. This document also describes a process for extracting a polypeptide composition from intercellular spaces of plants by infiltrating leaves with an extraction medium without rupturing the plant cells.

WO 94/03617 discloses the production of AFPs from yeast and their possible use in ice-cream. WO 96/11586 describes fish AFPs produced by microbes.

5 Up till now, however the use of AFPs has not been applied to commercially available consumer products. One reason for this are the high costs and complicated process for obtaining AFPs. Another problem is that sources of the AFPs are either difficult to obtain in sufficient quantities (e.g. fish  
10 containing AFPs) or are not directly suitable for use in food products.

The present invention aims to provide novel antifreeze polypeptides which have the advantage that they can easily be  
15 obtained from an abundant natural source and which provide good properties to products in which they are used.

It has been found that antifreeze polypeptides which possess good recrystallisation inhibition properties can be obtained  
20 from carrots. In particular it has been found that antifreeze polypeptides obtained from carrots show markedly better properties as compared to polypeptides isolated from other root vegetables. In particular the antifreeze polypeptides of the invention are capable of providing good recrystallisation  
25 inhibition properties without significantly changing the crystal shape of the ice-crystals, therewith possible leading to more favourable properties e.g. soft ice-cream.

Applicants have found that the effective antifreeze  
30 polypeptides from carrots are generally characterised by an apparent Molecular Weight on SDS-PAGE of 36 kDa. Accordingly in a first aspect the invention relates to antifreeze

In this context it will be clear to the skilled person that  
5 due to variation e.g. in SDS PAGE, the apparent molecular  
weight can only be determined with some variation in the  
results. For the purpose of the invention these variations  
e.g. from 30 to 40 kDa or from 34 to 38 kDa are also embraced  
within the scope of the term "apparent Molecular Weight of 36  
10 kDa".

Applicants also have found that the effective anti-freeze polypeptides according to the invention comprise fragments having an amino acid sequence as represented in the 15 examples.

Accordingly in a second aspect the invention relates to polypeptides comprising one or more fragments (A-E) having an amino acid sequence as follows:

20

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

25 (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-  
PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

30 (E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-  
LYS

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The complete amino acid sequence of the preferred AFP of the invention is represented below. Accordingly, in a third aspect the invention relates to an anti-freeze protein having an amino acid sequence as shown in Listing 1:

10  
a ATGAATATTGAATCATCTTTCTGCCCTATTTTGTGCATATGCATGATTTTCCTCTGCCCTT  
13 ----- 72  
M N I E S S F C P I L C I C M I F L C L -

15  
a CCAAACCTCTCTGCATCACAAAGATGCAACAACAACGACAAGCAAGCTTTACTCCAAATC  
73 ----- 132  
P N L S A S Q R C N N N D K Q A L L Q I -

20  
a AAAACAGCCTTGAAAAACCCACCATTACAGACTCATGGGTGTGAGACGACGATTGTTGT  
133 ----- 192  
K T A L K N P T I T D S W V S D D D C C -

25  
a GGTGGGACCTAGTCGAATGTGACGAAACCAGCAACCGCATAATTTCCCTCATAATTCAA  
193 ----- 252  
G W D L V E C D E T S N R I I S L I I Q -

30  
a GACGACGAAGCTCTCACCGGCCAAATCCACCTCAGGTGGGAGACCTACCATACCTCCAA  
253 ----- 312  
D D E A L T G Q I P P Q V G D L P Y L Q -

35  
a GCCTTATGGTTCCGTAAACTCCCAATCTTTTCGGAAAAATCCAGAAGAAATTTCTGCA  
313 ----- 372  
A L W F R K L P N L F G K I P E E I S A -

40  
a CTCAAAGACCTAAAATCCCTCAGACTCAGCTCGACCAGTCTCAGTGGCCCTGTCCCTTTA  
373 ----- 432  
L K D L K S L R L S S T S L S G P V P L -

45  
a TTCTTCCCTCAGCTTACGAAACTAACTTGTTAGACTTATCGTTTAACAAACTTTTGGGT  
433 ----- 492  
F F P Q L T K L T C L D L S F N K L L G -

## 40

Also embraced within the invention are isoforms and derivatives of the above mentioned polypeptides which still possess the antifreeze properties. Preferable the derivatives show at least 75% homology with the polypeptide of Listing 1 or the polypeptide comprising the partial sequences (A-E),

more preferred more than 85%, most preferred more than 95%.  
For the purpose of the invention the term derivative also  
embraces modified polypeptides which still possess the  
antifreeze properties, for example glycosylated forms of the  
5 above polypeptides.

Also embraced within the invention are nucleotide sequences  
encoding the amino acids as described above. In particular  
the invention relates to nucleotide sequences of Listing 1  
10 and alleles thereof.

Also embraced within the invention are nucleotide fragments  
derived from the coding region that are capable of  
hybridizing to related genes that code for anti-freeze  
15 peptides.

Although the proteins of the invention can easily directly be  
isolated from carrots, also genetic manipulation techniques  
may be used to produce the proteins described in the  
20 invention.

An appropriate host cell or organism would be transformed by  
a gene construct that encodes the desired polypeptide. The  
nucleotide sequence coding for the polypeptide can be  
25 inserted into a suitable expression vector containing the  
necessary elements for transcription and translation and in a  
manner that they will be expressed under appropriate  
conditions (eg in proper orientation and correct reading  
frame and with appropriate targeting and expression  
30 sequences). The methods required to construct these  
expression vectors are well known to those skilled in the  
art.

A number of expression systems may be utilised to express the polypeptide coding sequence. These include, but are not limited to, bacteria, yeast, insect cell systems, plant cell culture systems and plants all transformed with the appropriate expression vectors. Yeast, plants and plant culture systems are preferred in this context.

A wide variety of plants and plant cell systems can be transformed with the nucleic acid constructs of the polypeptides. Preferred embodiments would include, but are not limited to, maize, tomato, tobacco, carrots, strawberries, rape seed and sugar beet.

One preferred embodiment of the invention relates to the use of AFPs of the invention for increasing the frost tolerance of plants. This case for example be done by the above method whereby the plants are transformed to ensure (increased) production of the AFPs of the invention, therewith increasing the frost tolerance of said plants.

The invention also relates to antibodies which specifically bind an (epitope of the) polypeptides of the invention. Also embraced are polypeptides which are immunologically related to the polypeptides as determined by its cross reactivity with an antibody raised against the above polypeptides.

Based on the above information it is also possible to genetically modify other natural sources such that they produce the advantageous AFPs as identified here-above.

Preferably those AFPs are chosen which have significant ice-recrystallisation inhibition properties. A suitable test for determining the recrystallisation inhibition properties is indicated in example I. Also preferably AFPs in accordance to 5 the invention provide a ice particle size in the frozen product (mean crystal length) upon recrystallisation of less than 50  $\mu\text{M}$ , more preferred from 5 to 40  $\mu\text{m}$ .

The AFPs can conveniently be used in several products, 10 preferably in food products which are frozen or intended to be frozen. Carrots which comprise the AFP at naturally occurring levels are not embraced within the scope of the invention. However, food product containing (parts) of carrots are embraced within this term. Also embraced are 15 carrots which have been transformed to over express the AFP of the invention i.e. which contain the AFP at significantly higher levels than non-transformed carrots.

Examples of such food products are: frozen food products such 20 as vegetables, sauces, soups, snacks, frozen confectionery products such as ice-cream or water-ice, dairy products etc.

The preferred products wherein the AFPs are used are or frozen vegetables or frozen confectionery products such as 25 ice-cream or water-ice. Preferably the level of AFPs is from 0.00001 to 0.5 wt% based on the final product.

If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final 30 frozen product is within the above ranges. Surprisingly it has been found that compositions of the invention can contain very low amounts of AFPs while still being of good quality.



5

10 Also it is possible to modify the food product such that the  
AFP is produced in situ e.g. by adding genetically modified  
micro-organisms which are capable of producing the AFP in the  
food product, or even to genetically modify the food product  
(e.g. the vegetable) such that (the vegetable) in itself it  
15 is capable of producing the AFP in situ.

20 sorbet, ice milk and frozen custard, water-ices, granites and frozen fruit purees.

25 preferred from 10 to 70wt, for example 40 to 70 wt%.

30 ingredients of the formulation are fully mixed before the freezing process starts.

## EXAMPLES

Example I

- 5 Carrots (*Daucus carota* cv Autumn King) were grown in individual pots. When plants were approximately twelve weeks old, they were transferred to a cold room and held at 4°C in constant light during 4 weeks for cold-acclimation. Plants were watered three times a week.
- 10 Fresh tissue of the carrots were ground with a pestle and mortar (cooled to 4°C) in an equal volume buffer A (10mM EDTA, 20 mM Ascorbic acid, buffered with Tris to pH 7.4) held on ice. The homogenates were filtered through one layer of 15 muslin and kept on ice prior to further use.
- As a comparison several other root-plants were grown and homogenates prepared from the roots as above.
- 20 Anti-freeze activity was measured using a modified "splat assay" (Knight et al, 1988). 2.5 µl of the solution under investigation in 30% (w/w) sucrose was transferred onto a clean, appropriately labelled, 16 mm circular coverslip. A second coverslip was placed on top of the drop of solution 25 and the sandwich pressed together between finger and thumb. The sandwich was dropped into a bath of hexane held at -80°C in a box of dry ice. When all sandwiches had been prepared, sandwiches were transferred from the -80°C hexane bath to the viewing chamber containing hexane held at -6°C using forceps 30 pre-cooled in the dry ice. Upon transfer to -6°C, sandwiches could be seen to change from a transparent to an opaque

appearance. Images were recorded by video camera and grabbed into an image analysis system (LUCIA, Nikon) using a 20x objective. Images of each splat were recorded at time = 0 and again after 30-60 minutes. The size of the ice-crystals in both assays was compared. If the size at 30-60 minutes is similar or only moderately increased (say less than 20% increased, more preferred less than 10% increased, most preferred less than 5 % increased) compared to the size at t=0, this is an indication of good ice-crystal recrystallisation inhibition properties.

Results: from the sandwich splat assay test it appeared that samples from carrot roots, carrot stem and carrot leaves possess significant ice-recrystallisation inhibition properties, whereby the roots and leaves are most active. As a comparison a sample of non-acclimated carrot roots was tested, which showed significant less activity. For the following examples root tissue was used for further testing on carrots.

20

As a comparison several other vegetable roots were investigated by means of the sandwich splat assay test in 30% sucrose. Among these vegetables were turnip, kale, brussels sprout, wintergreen cabbage, rape, pak choi, parsnip and strawberry. None of these sources of material provided significant ice-recrystallisation inhibition activity.

Example II

Carrot root tissue was homogenized in three volumes (w/v) buffer (20mM ascorbic acid, 10 mM EDTA, 50 mM Tris/HCL, pH 7.2) in a pre-cooled pestle and mortar and filtered through one layer of muslin. The filtrate was centrifuged at 6,000 g, ten minutes at 4°C; the supernatant was collected and centrifuged at 100,000g for 1 hour at 4°C. The 100,000 g supernatant from this step is termed the soluble fraction and the pellet the microsomal fraction.

The supernatant was applied to a 30 ml fast flow Q Sepharose (Pharmacia) column pre-equilibrated in 50 mM Tris/HCL pH 7.4 at a flow rate of 5 ml/min supplied by a HiLoad pump P-50 controlled by a Gradifrac low pressure chromatography system (Pharmacia) at 4°C and the eluate monitored at OD 280 by a UV monitor (Monitor UV1, Pharmacia) recorded on a chart recorder (REC 102, Pharmacia). 5 ml fractions were collected. The column was washed with 50mM Tris/HCL pH 7.4 at the same flow rate until the OD 280 returned to zero. A 150ml gradient of 0-0.4 M NaCl in Tris/HCL pH 7.4 was then applied followed by a 2 M NaCl column wash. Eluate fractions were subjected to the splat assay as in example I.

Fractions containing anti-freeze activity as evidenced by recrystallisation inhibition were pooled and concentrated using polyethylene glycol as follows: the fractions were transferred in 10kDa cut off dialysis tubing (Sigma) which had been washed in tap water, boiled in 50mM EDTA pH 7.5 for 10 minutes and rinsed in milli Q water. The dialysis tubing containing the sample to be concentrated was covered with solid polyethylene glycol compound Mol. Wt. 15,000 - 20,000

(Sigma) and incubated at 4°C for up to 4 hours or until the sample volume inside the dialysis tubing had reduced up to 10 fold.

- 5 The pooled concentrate from the Q sepharose column was applied either to a phenyl Sepharose column, a SMART superdex 75 gel permeation column or an FPLC superdex 75 gel permeation column.
- 10 Carrot root anti-freeze proteins were purified by gel permeation chromatography as follows:
- 20  $\mu$ l aliquots of sample were applied to a SMART superdex 75 column (Pharmacia) pre-equilibrated in 50mM Tris/HCl pH7.4 containing 0.15M NaCl (Buffer E) at a flow rate of 40  $\mu$ l/min and components separated by gel permeation at the same flow rate in equilibration buffer. The eluate was monitored at OD 280 and OD 215. 80  $\mu$ l fractions were collected between 0.85 and 0.89ml, 40  $\mu$ l fractions between 0.89 and 1.24ml and 100  $\mu$ l fractions between 1.24 and 3.0 ml. The void volume ( $V_o$ ) of the column was 0.91 ml as determined by the retention volume of a solution of Blue Dextran. The superdex column was calibrated by application of 10  $\mu$ l of a solution containing 5mg/ml BSA ( $M_r$  66kDa, retention ( $V_e$ )=1.02 ml), 3mg/ml Carbonic anhydrase ( $M_r$  29 kDa,  $V_e$ =1.22 ml), 2mg/ml Cytochrome C ( $M_r$  12.4 kDa,  $V_e$ =1.41 ml) and 2mg/ml Aprotinin ( $M_r$  6.5 kDa,  $V_e$ =1.59 ml) and a standard curve plotted of  $V_e/V_o$  against log  $M_r$ . Fractions containing anti-freeze activity were identified by the splat assays as described in Example I, with an activity peak that showed a retention volume of 1.16 ml and an apparent molecular weight of 40 kDa. These measurement

confirmed that the 36 kDa band from cold acclimatised carrots was an anti-freeze peptide.

SDS-PAGE was carried out according to Laemmli (1970) using the Biorad mini system. Samples to be analyzed by SDS-PAGE were dissolved in SDS-PAGE sample buffer (Laemmli 1970), heated for 5 minutes at 100°C on a dry heating block (Techne) and centrifuged for 3 minutes at 10,000g at room temperature. Samples (10-50µl) were applied to mini-gels (Biorad, 0.75, 1.0 or 1.5mm thickness, 10, 12, 15% acrylamide or 10-20% gradient acrylamide (pre-poured from Biorad)) and electrophoretically separated. Separated polypeptides were fixed and stained in the gel either with Coomassie blue (0.1% (w/v) Coomassie Brilliant Blue in acetic acid/methanol/miliQ water (5:4:31, by vol)) or silver stained using the Biorad silver stain kit according to the manufacturer's instructions. Gels were dried between two sheets of Gelair cellophane in a Biorad gelair dryer according to the manufacturer's instructions. Sigma high and low range molecular weight marker kits were used according to the manufacturer's instructions for determination of apparent  $M_r$  on SDS-PAGE.

The ion exchange chromatography was carried out with cold acclimatised carrot root and non-cold acclimatised carrot root. The resulting gel SDS-PAGE gels showed the presence of a 36kDa band in the cold acclimatised sample. This band was much less abundant in the non-cold acclimatised root. This 36kDa band was hence attributed to anti-freeze activity.

Example III

For protein sequencing, the 36kDa carrot root protein was purified as described in the previous example and then to ensure further purification the sample to be sequenced was excised from the SDS PAGE gel and then proteolytically digested *in situ* in the polyacrylamide gel slice.

Preparations of largely pure 36 kDa protein, that still had some minor contaminating proteins, were loaded onto a 12% polyacrylamide gel. Three lanes each with 2 µg of protein were loaded and electrophoresed in the gel until the dye front reached the bottom of the gel. The gel was then stained in 0.2% uocmassie brilliant blue (w/v), 30% methanol (v/v), 1% acetic acid (v/v) for 20 minutes and then destain with 30% methanol until the protein bands could be visualised. The 36 kDa band was identified by comparison with molecular weight markers loaded into adjacent lanes and the band from each lane was excised with a scalpel blade, taking care to exclude contaminating bands.

The gel slices were transferred to a clean eppendorf tube and washed twice with 0.5ml of 50% acetonitrile (v/v), 100mM Tris/Cl, pH 8.5. The washing removed some of the uocmassie stain and also partially dehydrated the gel slices. The gel slices were then removed from the tube and subjected to air drying on the laboratory bench until they had shrunk significantly and started to curl up. They were then transferred back to the eppendorf and rehydrated with firstly, 10µl of 100mM Tris/Cl, pH 8.5 containing 1µg of endoproteinase Lys C (Boehringer Mannheim). This is a proteinase that specifically cleaves polypeptide chains on





Several polypeptide fragments (A-E) were analyzed in the 36 kDa band and had sequences substantially homologous to:

5

(A) \ LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

10 (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-  
PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

15 (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-  
LYS

**SECRET**

Sub 3

### Example IV-a carrot cell culture

5 University of Leeds. The culture was maintained by subculturing 10 ml of the culture into 90 ml of fresh Murashige and Skoog medium (Sigma) containing 25 g/l sucrose and 1 mg/l 2,4-D every seven days. Cultures were incubated in an orbital shaking incubator at 150 rpm at 25°C in the dark.

10

The NOR culture was cold treated as follows:

NOR cultures were transferred to 4°C after 4d and 7d of growth at 25°C. Cultures were harvested at t=0, t=7d and 15 t=14d. In addition to harvesting, the packed cell volume (PCV) was determined for each culture at each time point.

The media samples from NOR suspension cultures were analyzed as follows. Approximately 1/10th of the volume of a frozen aliquot of conditioned suspension culture medium was allowed to defrost. The defrosted (freeze concentrated) portion was removed and tested for activity by sandwich splat assays as described in Example I. Medium from cold acclimated cultures was found to contain significantly more activity than medium from non-cold acclimated cultures.

The cold acclimated NOR carrot medium was buffered by addition of 100 $\mu$ l of 1M Tris/HCl pH 7.4. Purification of activity was then performed by ion exchange and gel permeation chromatography using a method based on that in Example II: the buffered medium was applied to a 1 ml Q Sepharose column (Pharmacia) at a flow rate of 1 ml/min and

bound molecules eluted with 3 ml aliquots of 500 mM Tris/HCl pH 7.4 containing concentrations of NaCl starting at 0.1 M and increasing to 0.5 M in 0.1 M steps. 1 ml fractions were collected and tested for activity as in Example I.

5

The antifreeze activity in the active ion exchange fractions was purified by gel permeation chromatography as follows. The active fraction from above was acetone precipitated and the pellet resuspended in 50µl 50mM Tris/HCl + 0.15 M NaCl, pH 10 7.2. This was then centrifuged at 10.000 g for 10 minutes, and 20µl loaded onto a Superdex 75 gel permeation column on the Pharmacia SMART system. The flow rate was 40µl/min and the mobile phase was 50mM Tris/HCl + 0.15M NaCl, pH 7.2. 80µl fractions were collected and splatted. Activity was detected 15 in fractions corresponding to a retention of 1.16 ml.

Further isolation of the active proteins can be done by SDS PAGE analysis in line with Example II.

### Example IV-b carrot root culture

Carrot root cultures were initiated as follows.

- 5 For each individual culture 10 surface sterilised *Daucus*  
carote cv Autumn King seeds were placed into 100 ml MS medium  
containing 25 g/L sucrose and 0.5 g/L MES in sterile 250 ml  
Erlenmyer flasks. Seeds were germinated by shaking at 150 rpm  
in the dark at 25°C for 3 weeks. Leaves and shoots were then  
10 aseptically removed. The roots were replaced into 100 ml  
fresh medium and incubated with shaking for a further 2  
weeks.

Homogenates were prepared from cold treated and non-cold  
15 treated root cultures as follows. Fast frozen roots were  
ground up 3x in liquid nitrogen in a cold mortar and pestle  
then transferred to a further chilled mortar and pestle and  
ground up with 0.5x volume of ice-cold 50mM Tris.HCl + 10 mM  
EDTA pH 7.4 containing 30 % w/w sucrose. Homogenates were  
20 centrifuged at 10.000 g for 10 minutes at 4°C and the  
supernatant tested for activity as in Example I.  
Significantly more activity was detected in cold treated root  
cultures than in non-cold treated root cultures.

1. The first step is to identify the problem or question that needs to be addressed. This involves understanding the context and the specific requirements of the task.

Example V preparation of ice-cream

Root extract from cold acclimatised carrot roots was prepared by scrubbing freshly pulled cold acclimatised (as in example 5 I) carrots in cold water. The tops are removed and the juice extracted employing a domestic juice extractor (Russell Hobbs, model no 9915). The juice was frozen in 1 litre blocks and stored at -20°C prior to collection for use in ice cream trials. The carrot AFP juice was added to the following ice cream formulation:

INGREDIENT	parts by weight
Skimmed Milk Powder	10.000
Sucrose	13.000
MD40	4.000
Locust Bean Gum	0.144
Genulacta L100	0.016
MGP	0.300
Butteroil	8.000
Vanillin	0.012
Water	64.528
Carrot Extract (from cold acclimated carrots containing 1-10 mg AFP per kg)	4.472

Ice-cream was prepared by freezing the above formulation and aeration to 106% overrun.

Measurements were made on fresh sample and on samples which had been abused by storage at  $-10^{\circ}\text{C}$  for a period of 10 days. As a comparison a sample without carrot extract was measured in the same way. The measurements were done as follows:

Samples were equilibrated at  $-18^{\circ}\text{C}$  in a Prolan Environmental cabinet for approximately 12 hours. Three samples were chosen representatively from each batch of ice cream and a slide was prepared from each in a Cryostat temperature control cabinet by smearing a thin layer of ice cream from the centre of each block onto a microscopic slide. A single drop of white spirit was applied to the slide and a cover slip was then applied. Each slide, in turn, was then transferred to a temperature controlled microscope stage (Leit LaborLux S, Leica x10 objective, temperature  $-18^{\circ}\text{C}$ ). Images of ice-crystals (about 400 individual ice-crystals) were collected and relayed through a video camera (Sanyo CCD) to an image storage and analysis system (LEICA Q520MC).

The stored ice crystal images were highlighted manually by drawing around the perimeter which then highlights the whole crystal. Images of the highlighted crystals were then measured using the image analysis software which counts the number of pixels required to complete the longest straight line (length), shortest straight line (breadth), the aspect ratio (length/breadth). The data for each individual ice crystal of a batch of ice cream was imported into a spreadsheet where analysis of the data set was carried out to find the mean, and standard deviation.

The ice cream block was removed from Prolan temperature control cabinet and placed the Hounsfield H10KM Universal  
10 Tester. The 10cm cylindrical probe was pushed into the ice cream block at a constant rate of 400mm/min to a depth of 20mm. The maximum force recorded during the compression was used and expressed as the ice cream Hardness. If cracking or brittle fracture of the sample was observed this was  
15 indicated in the right hand column

The following results were obtained

Sample	Ice Crystal Size Parameters				Material Properties	
	Mean Crystal Length / $\mu\text{m}$	Mean Crystal Breadth / $\mu\text{m}$	Mean Crystal Shape Factor / -	Mean Crystal Aspect Ratio / -	Hardness / N	Brittle Fracture observation
Carrot AFP - fresh	26.79 $\pm$ 1.3	19.00 $\pm$ 0.9	1.15 $\pm$ 0.013	1.43 $\pm$ 0.024	40.8	Yes
Carrot AFP - Abused	33.48 $\pm$ 1.3	24.61 $\pm$ 0.9	1.13 $\pm$ 0.013	1.37 $\pm$ 0.020	59.9	Yes
Cont.- Fresh	33.67 $\pm$ 1.1	24.79 $\pm$ 0.8	1.12 $\pm$ 0.008	1.38 $\pm$ 0.018	27.3	No
Cont.- Abused	61.77 $\pm$ 2.7	46.54 $\pm$ 2.0	1.11 $\pm$ 0.010	1.37 $\pm$ 0.020	32.7	No

5 This proves that carrot AFP has good ice recrystallisation inhibition properties.

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Example VI

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The peptide sequences shown in Example III were analyzed as to their suitability for degenerate oligonucleotide primer design. Part of Peptide D (GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO) was chosen and the primer cp3 (GGI CCI GTI CCI YTI TTY TTY CC, where I= inosine and Y=C or T) was synthesized (Genosys).

- Sub BS
- 10 First strand cDNA was prepared from 5 µg cold acclimated (1 month as in example I) carrot root RNA using Superscript Reverse Transcriptase (Stratagene) and an oligonucleotide primer OG1 (GAGAGAGGATCCTCGAG(T)<sup>15</sup>) according to the manufacturer's instructions. 1% of the first strand cDNA
- 15 reaction was used as template, together with cp3 and OG1 primers, in subsequent PCR. The reactions were carried out in a thermal cycler using Taq DNA polymerase (Gibco BRL) for 30 cycles (1 minute at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C) according to the manufacturer's
- 20 instructions. All primers were used at a concentration of 1 µM. The resulting ~800 bp PCR product was gel purified and cloned into the pTAg vector (R&D Systems) according to the manufacturer's instructions. The cloned cp3 PCR product was sequenced using the dideoxy sequencing method employed by
- 25 the Sequenase kit (USB). The cp3 nucleotide sequence and deduced amino acid sequence were substantially similar to:

5 a 1 GGGCCGGTGCCGCTGTTCTTCCCTCAGCTTACGAAACTAACTTGTTTAGACTTATCGTTT 60  
G P V P L F F P Q L T K L T C L D L S F -  
AACAAACTTTTGGGTGTAATCCCTCCTCAGCTTTCCTCCTCCGAACCTTAAAGCCCTG  
61 a 120  
N K L L G V I P P Q L S T L P N L K A L -  
10 CACTTAGAACGTAACGAACTCACCGGTGAAATCCCGATATCTTTGGGAATTTTGTCTGGA  
121 a 180  
H L E R N E L T G E I P D I F G N F A G -  
15 TCCCCGGACATATATCTTTTCGCATAACCAGCTCACCGGGTTTGTTCCTCCAACTTTTGTCT  
191 a 240  
S P D I Y L S H N Q L T G F V P K T F A -  
20 AGAGCAGATCCAATTAGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTCATTC  
241 a 300  
R A D P I R L D F S G N R L E G D I S F -  
25 TTGTTTGGGCCTAAAAACGCTTGGAAATGCTAGATTTTTCAGGAAACGTGCTTAGTTTC  
301 a 360  
L F G P K K R L E M L D F S G N V L S F -  
AATTTCTCCAGGGTGCAGGAGTTTCCACCCTCTTGACATACTTAGACTTGAACCATAAC  
361 a 420  
N F S R V Q E F P P S L T Y L D L N H N -  
30 CAGATCAGCGGAAGTCTGTGAGTGAATTGGCTAAATTGGACCTGCAGACATTTAACGTC  
421 a 480  
Q I S G S L S S E L A K L D L Q T F N V -  
35 AGTGATAATAATCTCTGCGGCAAGATTCCAACAGGGGGAAACCTCCAGAGATTGACCGT  
481 a 540  
S D N N L C G K I P T G G N L Q R F D R -  
40 ACGGCTATCTCCACAACAGTTGCTTGTGTGGTGTCCATTGCCAGAATGCTAGTTACCA  
541 a 600  
T A Y L H N S C L C G A P L P E C \*  
TGCAAAATGTGCCTTAAGGTTATCTTTGTAATGAGATATATTATGCAGCTCAAGGCAGAG  
601 a 660

10

Positive plaques were screened to purity and phage-mids  
25 excised before the inserts were characterised by DNA  
sequence analysis. Two cDNA clones were sequenced to  
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Positive plaques were screened to purity and phage-mids  
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sequence analysis. Two cDNA clones were sequenced to  
completion. Although the 5' and 3' untranslated regions  
contained some sequence variability, the coding regions  
were identical. The coding regions of the two cDNA clones  
30 were substantially similar to:

5 a 13 ATGAATATTGAATCATCTTTCTGCCCTATTTTGTGCATATGCATGATTTTCCTCTGCCTT 72  
 M N I E S S F C P I L C I C M I F L C L -  
 a 73 CCAAACCTCTCTGCATCACAAGATGCAACAACACGACAAGCAAGCTTTACTCCAAATC 132  
 P N L S A S Q R C N N N D K Q A L L Q I -  
 10 a 133 AAAACAGCCTTGAAAAACCCACCATTTACAGACTCATGGGTGTCAGACGACGATTGTTGT 192  
 K T A L K N P T I T D S W V S D D D C C -  
 15 a 193 GGTGTTGGACCTAGTCGAATGTGACGAAACCAGCAACCGCATAATTTCCCTCATAATTCAA 252  
 G W D L V E C D E T S N R I I S L I I Q -  
 20 a 253 GACGACGAAGCTCTCACCGGCCAAATCCCACCTCAGGTGGGAGACCTACCATACCTCCAA 312  
 D D E A L T G Q I P P Q V G D L P Y L Q -  
 a 313 GCCTTATGGTTCCGTAAACTCCCAATCTTTTCGAAAAATCCCAGAAGAAATTTCTGCA 372  
 A L W F R K L P N L F G K I P E E I S A -  
 25 a 373 CTCAAAGACCTAAATCCCTCAGACTCAGCTCGACCAGTCTCAGTGGCCCTGTCCCTTTA 432  
 L K D L K S L R L S S T S L S G P V P L -  
 30 a 433 TTCTTCCCTCAGCTTACGAAACTAAGTTGTTTAGACTTATCGTTTAACAACTTTTGGGT 492  
 F F P Q L T K L T C L D L S F N K L L G -  
 35 a 493 GTAATCCCTCCTCAGCTTTCCACTCTTCCGAACCTTAAAGCCCTGCACTTAGAACGTAAC 552  
 V I P P Q L S T L P N L K A L H L E R N -  
 40 a 553 GAACTCACCGGTGAAATCCCGATATCTTTGGGAATTTTGCTGGATCCCGGACATATAT 612  
 E L T G E I P D I F G N F A G S P D I Y -  
 45 a 613 CTTTCGCATAACCAGCTCACCGGGTTTGTTCCTCCAAACTTTTGCTAGAGCAGATCCAATT 672  
 L S H N Q L T G F V P K T F A R A D P I -  
 a 673 AGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTTTATTCTTGTGTTGGGCCTAAA 732  
 R L D F S G N R L E G D I S F L F G P K -  
 50 a 733 AAACGCTTGAAATGCTAGATTTTTCAGGAAACGTGCTTAGTTTCAATTTCTCCAGGGTG 792  
 K R L E M L D F S G N V L S F N F S R V -  
 55 a 793 CAGGAGTTTCCACCCTCTTTGACATACTTAGACTTGAACCATAACCAGATCAGCGGAAGT 852  
 Q E F P P S L T Y L D L N H N Q I S G S -  
 60 a 853 CTGTCGAGTGAATTGGCTAAATTGGACCTGCAGACATTTAACGTCAGTGATAATAATCTC 912  
 L S S E L A K L D L Q T F N V S D N N L -  
 65 a 913 TGCGGCAAGATTCCAACAGGGGAAACCTCCAGAGATTCGACCGTACGGCCTATCTCCAC 972  
 C G K I P T G G N L Q R F D R T A Y L H -  
 a 973 AACAGTTGCTTGTGTGGTGTCTCCATTGCCAGAATGCTAG 1011  
 N S C L C G A P L P E C -

Partial sequence analysis of 4 other clones also indicated that they contained the same coding region as the fully sequenced clones and thus all the positives from the library screen were likely to represent transcripts from the same gene. The existence of only one copy of the AFP gene in the carrot genome was further substantiated by the fact that Southern analysis of restriction enzyme digested carrot genomic DNA suggested that only one fragment hybridized to the probe.

## Example VII

In order to prove that the carrot cDNA as shown in example 5 VI represented an AFP, expression of the coding region was carried out as follows. One of the cDNAs was first cloned into an intermediate pUC plasmid vector (Messing, 1983) containing a double CaMV 35S promoter (Guerineau, J. F., Woolsten, S., Brooks, L., and Mollineaux, P. (1988)) expression cassette, and then into a binary vector, as described below. All enzymes used were supplied by Gibco BRL and used according to the manufacturer's instructions.

The pBluescript phagemid (Stratagene) containing the cDNA 15 clone was digested with *Xho* I and the recessed 3' termini filled in using the Klenow fragment of DNA polymerase I. The cDNA fragment was then released from the vector by digestion with *Eco* RI. The *Eco* RI/blunt cDNA fragment was then cloned into the *Eco* RI/blunt digested intermediate pUC 20 plasmid vector. The CaMV 35S-cDNA expression cassette was then subcloned as a partial *Hind* III fragment into *Hind* III-cut pBin 19 binary vector (Bevan 1984). The binary vector construct was then introduced into tobacco using *Agrobacterium* mediated transformation (as described in 25 Draper, J., Scott, R., Armatage, P., and Walden, R. (1988)).

Transgenic tobacco callus was analyzed for expression of recrystallisation inhibition activity as soon as sufficient 30 kanamycin resistant material was regenerated. Small scale protein extracts were made from several independent

kanamycin resistant calli plus some wild type tobacco callus. Approximately 2 g tissue was ground up in 1-2 mls sucrose buffer (30 % sucrose 50 mM Tris, 10 mM EDTA, 20 mM ascorbate, pH 7.2) using a mortar and pestle.

5 The solution was centrifuged at 10,000 xg for 2 minutes and the supernatant removed to a fresh tube. An aliquot of 3 µl of protein extract was tested for recrystallisation activity using the sucrose sandwich splat assay method of example I. All kanamycin resistant callus extracts tested

10 demonstrated recrystallisation inhibition activity.

Stable transgenic tobacco plants expressing the carrot AFP have also been produced. Leaf extracts from wild-type and transgenic tobacco plants have been subjected to northern

15 analysis using the AFP cDNA as a probe. The AFP message was only detectable in the transgenic tobacco plants. This suggests that the AFP message is stable in the greenhouse grown transgenic tobacco plants. When compared with the native carrot transcript, the tobacco AFP transcript

20 appears to be slightly bigger. This discrepancy can be explained by the method of construction of the AFP expression cassette. Because the CaMV 35S polyadenylation signal is most 3' in the construct, it is likely that this signal is used in the transgenic AFP message, giving rise

25 to a longer transcript. Leaf extracts from wild-type and transgenic tobacco plants have also been analyzed by western blotting using a carrot AFP antibody. A cross-reacting protein was only detected in the transgenic tobacco plants. Despite the difference in transcript size,

30 the protein produced in tobacco appears to be the same size as the native carrot AFP.